Nucleoside-tailored Molecularly Imprinted Polymeric Nanoparticles (MIP NPs)

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Abstract: MIP NPs represent one of the current most suitable alternatives to antibodies for molecular recognition and diagnostic applications. Here we present the synthesis of MIP NPs imprinted for 2'-deoxyadenosine (dA) prepared using for the first time a modified polymerizable 2'-deoxyuridine complementary residue. We demonstrate that the introduction of this modified monomer results in an increase of the affinity of the produced MIP NPs, without altering their physical properties such as size, shape or dispersibility. The imprinted NPs have shown the ability to specifically recognize dA whereas no imprinting effect was observed for 2'-deoxyguanosine (dG) or deoxycytidine (dC). The results suggest that such monomers (and their phosphoramidites) could be used in the synthesis of oligomer or longer DNA sequences for potentially producing hybrid MIP-DNA materials with improved recognition performance.

1. Introduction

Nucleosides are a class of biomolecules of great biochemical importance. They are the building blocks of the genetic information codified in our DNA and RNA.[1] In their nucleotide form (tri- or diphosphate), they take part in energy distribution processes in cells, while their cyclic monophosphate analogues are key second messengers which regulate a wide variety of biological processes such as cellular growth and neuroplasticity.[2-4] Adenosine and its receptors play a significant role in Alzheimer's disease.[5] Moreover, modified nucleosides derived from RNA degradation in the organism were found to be excreted in urine at significantly elevated levels in patients with various cancers,[6, 7] AIDS, and a number of metabolic diseases.[8, 9] For these reasons, these compounds have been identified as potential markers for a number of disorders, and their specific detection represents an area currently under active development.

Different diagnostic tools may be used to qualitatively analyze and quantify these markers, such as antibody-based assays or other analytical techniques (e.g., LC-MS).[8-10] However, most of these procedures are quite laborious, requiring sample pre-concentration steps by solid-phase extraction (SPE) or other techniques, while other tools based on antibodies may suffer from difficult manufacturing,[11, 12] low stability, poor performance in non-physiological conditions,[13-16] and potentially short shelf-life.[17, 18] Additionally, natural antibodies can be difficult to integrate with assays and sensors using industrial protocols such as microfabrication and photolithography.[19, 20]

A possible alternative is the use of molecularly imprinted polymers,[21, 22] and there is a growing interest in the preparation of molecularly imprinted materials specific for nucleoside and nucleotide compounds.[23-26]

Given the advances in nanotechnology for biomedical and diagnostic applications,[27, 28] however, it is not surprising that in the last decade a more interesting approach towards the

formation of real artificial antibodies alternatives has been represented by MIP NPs,[29-32] which share key characteristics with their biomolecular counterparts such as aqueous solubility, size, affinity and selectivity for the target analyte.[33] Additionally, MIP NPs represent a convenient format for the development of sensors and assays, because solutions of these nanosystems can be handled similarly to solutions of antibodies, with the advantage of stability and robustness typically associated with these synthetic materials.[34, 35] Operational parameters for producing MIP NPs can be carefully controlled, thus obtaining synthetic receptors with homogenous population of binding sites which exhibit recognition properties similar to monoclonal antibodies.[36, 37]

In a recent example, authors imprinted adenosine in core-shell MIP sub-micrometer particles bearing a polymer layer on a silica core, but despite its elegance the synthetic procedure used in this work was quite long and complex, making it poorly scalable.[38, 39]

Here we present a simple method for the preparation of molecularly imprinted nanoparticles for dA recognition, combining two approaches. First, we have synthesized a tailored functional monomer based on the modification of a 2'-deoxyuridine (dU) nucleoside complementary to the dA template molecule. A C-5 alkene was added to make the dU residue polymerizable; this does not interfere with the base-pairing face so it can be used directly as a functional monomer in the imprinting process while still inducing specific attractive interactions with the dA template molecules. This is aimed at increasing the imprinting effect and the specificity of the MIP product. In addition, we have used a solid-phase imprinting technique to rapidly produce MIP NPs of low dispersity and uniform affinity, which can be difficult to achieve with other methods. Indeed, the solid-phase synthetic approach directly addresses these issues, resulting in MIP NPs with pseudo-monoclonal binding properties.[40, 41]

In comparison to other molecularly imprinted polymers produced for nucleosides and nucleotides, either as solid phases for pre-concentration purposes or as thin layers for sensing

applications,[23-26] the nanomaterials here presented are simple and quick to produce and, differently from other approaches,[38, 39] their production relies on a time-efficient and scalable method. The presence of the AcrU monomer directly embedded into the polymer matrix without the use of spacing sequences ensures the formation of high-affinity and high-specificity binding sites without need for complicated stepwise or layer-by-layer techniques.[38, 39] Moreover, one of the main advantages of materials prepared in this manner is the possibility of directly replacing antibodies with MIPs in standard analytical techniques or biosensors such as ELISA-like assays or biosensing measurements with minimal modification of the immobilization and analytical protocols.[42-44]

2. Experimental Section

Materials. *N*-isopropylacrylamide (NIPAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), acrylic acid (AAc), *N*,*N*′-methylenebisacrylamide (BIS), *N*-*tert*-butylacrylamide (TBAm), 3-aminopropyltriethyloxysilane (APTES), glutaraldehyde, sodium borohydride, cysteamine, methylacrylate, triethylamine (TEA), palladium(II) acetate, *N*,*N*-dimethylformamide (DMF), 4-methoxybenzaldehyde, glass beads, SPE cartridges and frits, toluene, methanol and acetone were purchased from Sigma-Aldrich (UK). Sodium hydroxide, chloroform, acetic acid, sulfuric acid and phosphate buffered saline (PBS) were purchased from Fisher Scientific (UK). Ethanol and hydrogen peroxide were purchased from VWR (UK). Deuterated NMR solvents and NMR tubes were purchased from Goss Scientific (UK). Nucleosides 2'-deoxyadenosine (dA), 2'-deoxyguanosine (dG), 2'-deoxycytidine (dC) and 5-iodo-2'-deoxyuridine were purchased from Link (UK). Double-distilled water (Millipore) was used for analysis. All chemicals and solvents were analytical or HPLC grade and were used without further purification.

Synthesis of 5-(2-carbomethoxyvinyl)-2'-deoxyuridine (AcrU). The synthesis was adapted from Ami and Fujimoto,[45] and carried out in a CEM-Discover monomode microwave apparatus (Frequency: 2.45 GHz). The temperature was measured with an IR sensor on the outer surface of the process vial. Briefly, 5-iodo-2'-deoxyuridine (1.00 g, 2.82 mmol) and $Pd(OAc)_2$ (0.06 g, 0.28 mmol) were suspended in DMF (3 mL) in a 10 mL glass vial equipped with a small magnetic stirring bar. To this suspension TEA (0.39 mL, 2.82 mmol) and methylacrylate (0.38 mL, 4.24 mmol) were added and the vial was tightly sealed with an aluminum/Teflon® crimp top. The mixture was irradiated for 4 min at 100 °C, using an irradiation power of 60 W. After the irradiation period, the reaction vessel was cooled rapidly (60-120 s) to 50 °C by gas jet cooling before it was opened. It was then filtered and evaporated under vacuum. The crude was purified by gradient flash chromatography ($CHCl_3$/MeOH, 95/5 to 90/10) on silica gel 60 (VWR). Fractions were analyzed using thin layer chromatography (TLC) and the product was visualized by UV absorption at 264 nm or by dipping in a solution of 4-methoxybenzaldehyde/AcOH/H_2SO_4/EtOH (5/1/1/50, v/v/v/v) and subsequent heating. After evaporation of the appropriate fractions under vacuum, 0.40 g of product were obtained (45%, mol/mol). ^{1}H-NMR ($CDCl_3$/CD_3OD, 9/1, 400 MHz, JEOL ECX 400): 8.39 ppm (s, 1H, H_6), 7.22 ppm (d, 1H, J = 15.9 Hz), 6.78 ppm (d, 1H, J = 15.9 Hz), 6.15 ppm (t, 1H, J = 6.2 Hz, H_1'), 4.35 ppm (m, 1H, H_3'), 3.88 ppm (m, 1H, H_4'), 3.83-3.79 ppm (m, 1H, H_5'), 3.70-3.62 ppm (m and sharp s, 4H, OCH_3 + H_5"), 2.32-2.26 ppm (m, 1H, H_2"), 2.15-2.09 ppm (m, 1H, H_2'). UV (H_2O, Perkin Elmer LAMBDA Bio+ Spectrophotometer): λ_{max} = 302 nm (ε = 17600).

Preparation of 2'-deoxynucleoside-derivatized glass beads as affinity media. Glass beads (125 g, 75 µm diameter, Supelco) were activated by boiling in NaOH (1 M) for 10 min, then washed thoroughly with double-distilled water at 60 °C, acetone and finally dried at 80 °C. They were then incubated in a solution of APTES (2 %, v/v, 0.8 mL solution/g glass

beads) in anhydrous toluene overnight at room temperature, then washed with acetone and subsequently incubated in a glutaraldehyde solution (7 %, v/v, 0.9 mL solution/g glass beads) in PBS (0.01 M, pH 7.2) for 5 h at room temperature, after which they were rinsed with double-distilled water. The dA, dG and dC templates were then immobilized by incubating the glass beads with a solution (5 mg/mL) of each template in PBS (0.01 M, pH 7.2) overnight at room temperature (0.67 mL solution/g glass beads). The derivatized beads were washed with double-distilled water and then reduced in a 1 mg/mL of $NaBH_4$ in acetate buffer (0.1 M, pH 4) for 30 min at room temperature (0.67 mL solution/g glass beads), then washed thoroughly with double-distilled water and dried under vacuum. After this step the glass beads were used straight away for the synthesis of the MIP NPs without further storage. The amount of template immobilized was determined spectrophotometrically (at λ_{max} = 260 nm for dA, λ_{max} = 253 nm for dG and λ_{max} = 270 nm for dC) by analyzing the amount of nucleoside unbound to the glass beads and found in the washings collected from both the immobilization and the reduction steps.

Solid-phase synthesis of dA, dC and dG Plain and AcrU MIP NPs. The following monomers were dissolved in H_2O (100 mL):[46] NIPAm (39 mg, 0.35 mmol, 53%), BIS (2 mg, 0.01 mmol, 2%), TBAm (33 mg, 0.26 mmol, 40%) and AAc (2.2 µL, 0.03 mmol, 5%). TBAm was previously dissolved in EtOH (2 mL) and then added to the aqueous solution. In the case of AcrU MIP NPs, the amount of NIPAm was reduced (35 mg, 0.32 mmol, 48%), TBAm was previously dissolved in 1 mL EtOH (rather than 2 mL) and AcrU (10 mg, 0.03 mmol, 5%) was added by previously dissolving it in 1 mL EtOH. The total monomer concentration was 6.5 mM for both Plain and AcrU MIP NPs. The solutions were degassed under vacuum and sonication for 10 min, and then purged with Ar for 30 min. After this time, 5 mL of each solution were transferred in a 14 mL glass vial closed using a Teflon screw-cap and containing 7.5 g of dA, dG or dC-derivatized glass beads (0.67 mL solution/g glass

beads), for a total of six polymerization vials (Plain MIP NPs for dA, dG, and dC; AcrU MIP NPs for dA, dG and dC). Prior to the addition of the polymerization mixture, the vials containing the solid phase were degassed under vacuum and the air inside the vials then replaced with Ar (3 times). The polymerization was started by adding an APS aqueous solution (50 µL, 60 mg/mL) and TEMED (1.5 µL). The polymerization was then carried out at 20 °C for 15-20 h. After the polymerization, the contents of the vials were transferred into SPE cartridges fitted with a polyethylene frit (20 µm porosity) in order to perform the temperature-based affinity separation of MIP NPs. The temperature of H_2O and the SPE cartridges was kept at 20 °C (same as the polymerization step). Washing was performed with 3 × 5 mL of H_2O, applying manual pressure with a syringe if needed. This was done in order to remove non-polymerized monomers and low-affinity MIP NPs. The effectiveness of the washing was verified by measuring the UV absorbance of washing aliquots, in order to ensure complete monomer removal as well as to quantify the incorporation of AcrU monomer into the polymer matrix (by difference of the absorbance measured at λ_{max} = 302 nm). Afterwards the SPE cartridges containing the solid phase with high-affinity MIP NPs attached were heated up to 60 °C and eluted with 5 × 5 mL H_2O at 60 °C. The concentration of the nanoparticles fractions has been evaluated by evaporation. Absence of nucleoside templates in the MIP NPs has been confirmed spectrophotometrically (at λ_{max} = 260 nm for dA, λ_{max} = 270 nm for dC and λ_{max} = 253 nm for dG).

Transmission Electron Microscopy (TEM) analysis. TEM images of MIP NPs were taken using a JEOL JEM 1400, 120kV high contrast TEM equipped with an AMT XR60 mid-mount digital camera (11 megapixels). Samples for the analysis have been prepared by depositing a drop of the MIP NPs solution, previously filtered through a 0.45 µm PTFE syringe filter, on a carbon-coated TEM copper grid (300 mesh, from Agar Scientific, UK), blotting away the excess and leaving them to dry overnight at room temperature.

Treatment of Quartz Crystal Microbalance (QCM) crystals and surface immobilization of templates. QCM crystals (5 MHz Cr/Au, polished, Testbourne Ltd., UK) were cleaned by immersion in Piranha solution (H_2SO_4/H_2O_2, 3/1, v/v) for 5 min. **Caution! This mixture is highly corrosive, hence extreme care is required during this process.** Then they were thoroughly rinsed with double-distilled water and left in MeOH overnight. The immobilization of the templates has been performed by incubating the crystals in a solution of cysteamine (0.2 mg/mL) in EtOH at 4 °C for 24 h, after which they have been washed with EtOH and incubated in a solution of glutaraldehyde (7 %, v/v) in PBS (0.01 M, pH 7.2) for 5 h at room temperature. After this step, the crystals were rinsed with double-distilled water and immersed in a 5 mg/mL solution of each template (dA, dC or dG) in PBS (0.01 M, pH 7.2) overnight at room temperature. Before analysis, the QCM crystals were rinsed with double-distilled water and then reduced in a 1 mg/mL solution of $NaBH_4$ in acetate buffer (0.1 M, pH 4) for 30 min at room temperature. Once the immobilization was completed, the crystals were washed thoroughly with double-distilled water before being mounted in the QCM flowcell. Successful immobilization of the templates was confirmed through sessile water contact angle measurements performed on a Theta Optical Thensiometer (Biolin Scientific, UK).

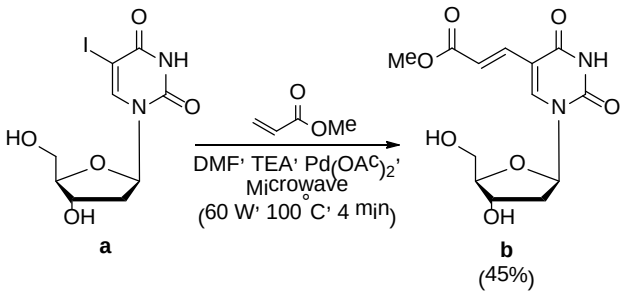
QCM microgravimetric analysis of Plain and AcrU MIP NPs. Plain and AcrU MIP NPs adsorption to dA, dC and dG templates was monitored using a QCM200 5 MHz quartz crystal microbalance (Stanford Research Systems, UK).[47] The modified QCM chips were maintained hydrated during mounting in the QCM flowcell. MIP NPs solutions and running buffer were introduced using an Instech P720 peristaltic pump equipped with 0.020” ID tubing (Linton Instrumentation, UK) and flowing at 0.1 μL/min. The QCM chip bearing the template was first stabilized in running buffer (PBS 0.01 M, pH 7.4) at 20 °C until the system reached a stable baseline. Affinity analysis was carried out by sequentially flowing each MIP

NPs solution for 5 min (500 µL) and analyzing the sensor response for 15 min. This process was repeated over the concentration range of 0.125–2 µg/mL. The specificity of the dA MIP NPs was assessed by cross-testing their affinity on all the chips prepared (bearing dA, dC or dG nucleoside template).

3. Results and discussion

Synthesis of 5-(2-carbomethoxyvinyl)-2'-deoxyuridine (AcrU). Several 5-substituted pyrimidine analogues have been investigated over the years and discovered as highly active antiviral, anticancer or antibacterial agents.[48-50] In this case, the palladium-catalyzed modification of 5-iodo-2'-dU (Scheme 1)[45] had the sole intent to introduce a polymerizable moiety suitable to prepare a chemical species capable of being introduced into a polymer matrix.

Scheme 1. Synthesis of 5-(2-carbomethoxyvinyl)-2'-deoxyuridine (AcrU, b) from 5-iodo-2'-deoxyuridine (a).



After preliminary experiments with different vinyl and allyl derivatives,[51, 52] we chose to perform this derivatization with methyl acrylate mainly for two reasons: a) the electron-withdrawing effect of the ester group should render the double bond reactive towards radical polymerization but at the same time not hindered enough to represent an obstacle either towards the introduction into the polymer nor the hydrogen bonding with the dA template species; b) the possibility of performing the reaction in a microwave-assisted environment

allowed to achieve rapidity, good yields and control over the experimental conditions.[45, 53] It was also noted that the allyl derivatives investigated were not incorporated into the polymer matrixes at a required level to achieve the nucleoside-NPs synthesis (data not shown).

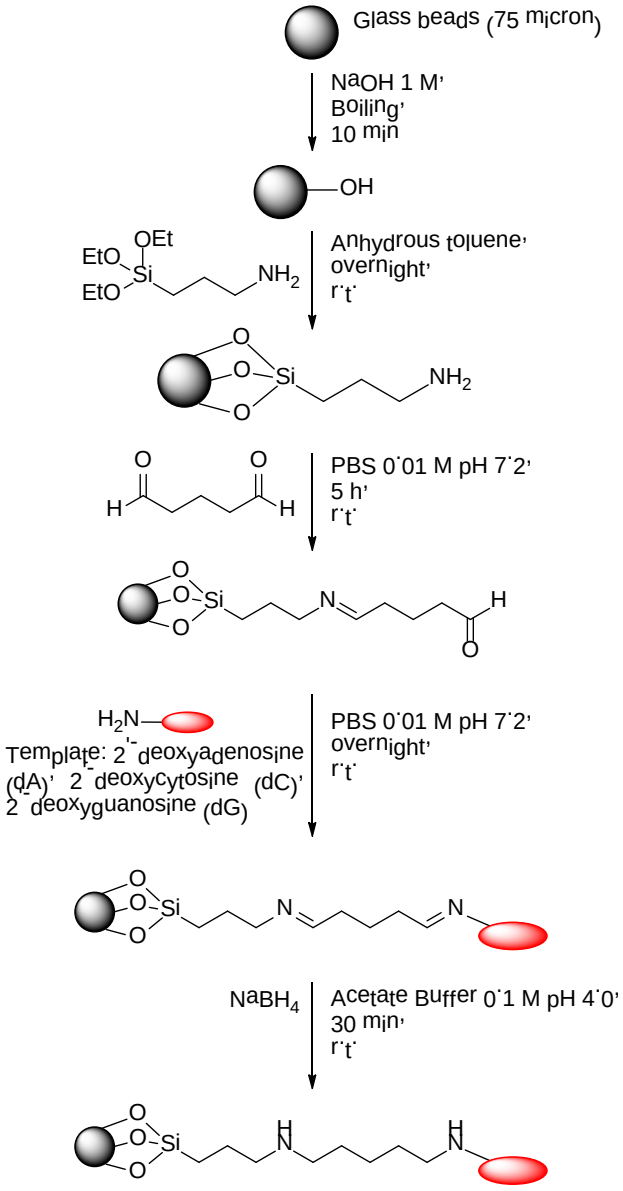
Since dU is a pyrimidine base complementary to dA, with which it forms hydrogen bonds, this should enhance dA adsorption within the polymeric matrix. Thus, the MIP product should be able to complex the compounds bearing dA moieties, resulting in a rational tailoring of the interactions between the template and the polymer material. Moreover, as a result of this specific interaction, the cross-reactivity with other nucleosides or related compounds should be reduced.

A similar approach had been adopted by Nowakowska *et al.*[38, 39] during the synthesis of core-shell imprinted sub-micrometer particles in which they built the imprinted shell using a layer-by-layer coating technique with a terpolymer containing thymine moieties. The authors demonstrated that the thymine chromophores underwent photodimerization when irradiated with UV light, thus allowing the photochemical molecular imprinting of the adenine template to take place. This is also the case for the tailored monomer here described, since its pyridine moieties should retain the ability to dimerize when irradiated with UV light, making such tailored monomer a potential multi-point cross-linker as well as template adsorbing center in the case of UV photopolymerization in a MIP matrix.

In the work of Nowakowska *et al.*,[38, 39] however, the authors did not modify the nucleoside structure but the nitrogenous base in position 1 (which usually in a nucleoside structure connects the base to the sugar portion). Differently from the approach here described their strategy, as well as the similar strategies of other authors not directly investigating imprinted polymers,[54-56] is poorly transferable to the possibility of exploiting larger and more complex species as monomers, such as oligomers or aptamer sequences.

Preparation of 2'-deoxynucleoside-derivatized glass beads as affinity media. The protocol used to prepare the solid-phase affinity media with dA, dC or dG as immobilized template is depicted in Scheme 2.

Scheme 2. Synthetic protocol for the immobilization of the templates (dA, dC or dG) on the glass beads surface.



The beads were activated in boiling NaOH solution to increase the number of reactive OH groups on their surface. This facilitates the following step, silanization with APTES, and results in a good coverage of the glass surface with silane containing primary amino groups

(potentially up to 12 µmol per gram of solid phase).[57] Glutaraldehyde is then added to act as a linker between the two primary amino groups (one on the glass surface, the other on the template) through the formation of imine bonds. The imines are stabilized by borohydride reduction in the subsequent step. This immobilization strategy is commonly used to attach enzymes or other proteins onto amino-bearing surfaces,[58] and has been similarly exploited in the case of immobilization of amino-functionalized templates in the imprinting of nano and microparticles due to its immediacy as well as efficacy.[29, 37, 40-43, 59-61] The spacing sequence resulting from the APTES and glutaraldehyde portions ensures enough accessibility to the template for the imprinting process to take place. The amount of nucleoside templates immobilized in this way, determined spectrophotometrically (at λ_{max} = 260 nm for dA, λ_{max} = 270 nm for dC and λ_{max} = 253 nm for dG) by analyzing the amount of nucleoside unbound to the glass beads, was 1.4 µmol/g of glass beads for dA, 1.1 µmol/g for dC and 0.5 µmol/g for dG.

Solid-phase synthesis of dA, dC and dG Plain and AcrU MIP NPs. The solid-phase synthesis of MIP NPs is then performed in the presence of template immobilized on glass beads. The principle behind this method is summarized in Figure 1.

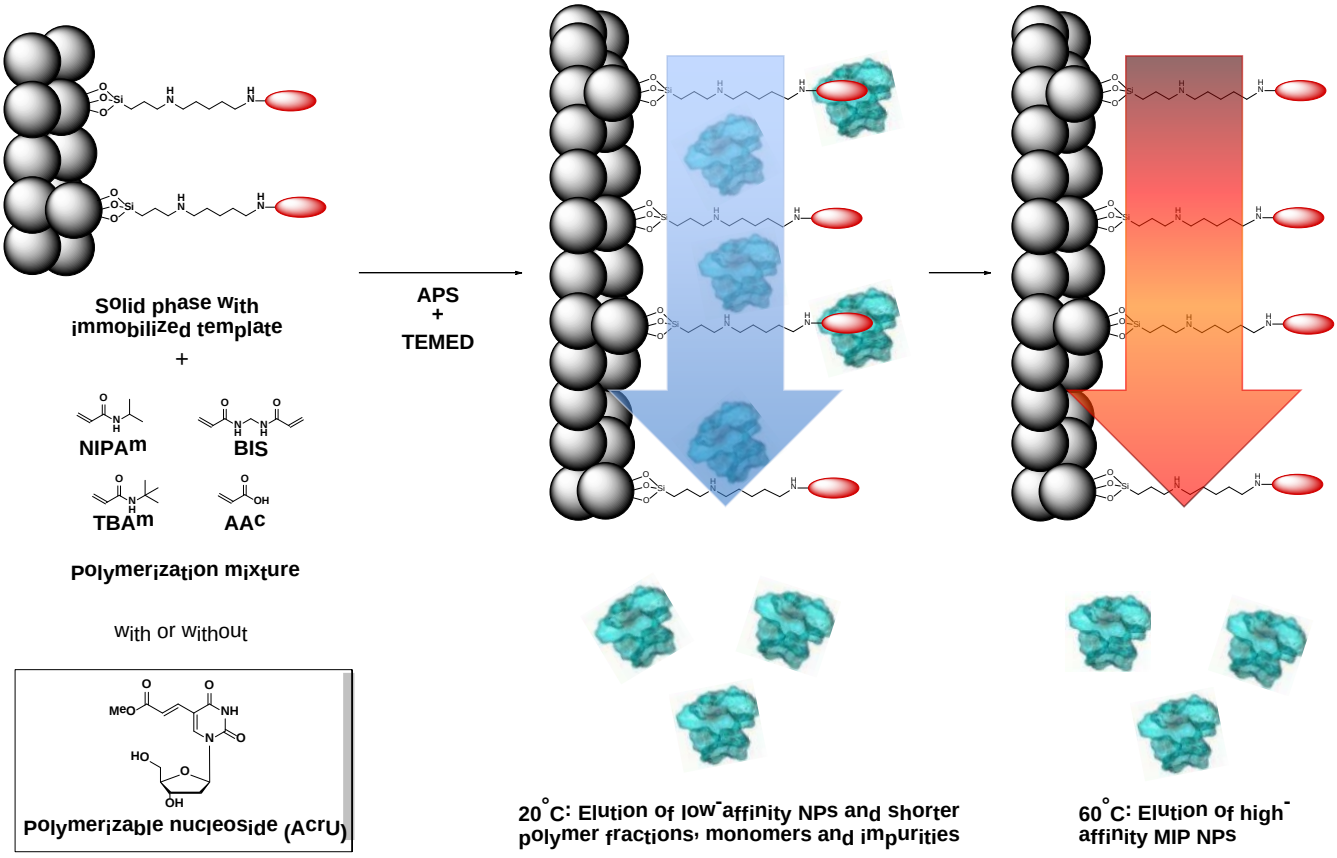


Figure 1. Schematic representation of the solid-phase synthesis and selection of Plain and AcrU MIP NPs. The monomers are mixed with the solid phase bearing the immobilized template (either dA, dG or dC) and the polymerization is initiated by the addition of APS and TEMED. In the case of AcrU MIP NPs, the polymerizable nucleoside is included in the polymerization mixture. The low-affinity particles, as well as unreacted monomers, are washed at relatively low temperature. The temperature is then increased and high-affinity MIP NPs particles are eluted from the solid phase for collection. Scheme adapted from Poma *et al.*[41]

As previously demonstrated,[40, 41] this solid-phase imprinting technique is extremely convenient for the production of high-affinity MIP NPs, offering several advantages in comparison with classical preparation procedures.[62] Indeed, this method produces only one fraction of template-free high-affinity MIP NPs with a narrow distribution of binding sites,

which are easily purified from low-affinity fractions and non-polymerized monomers and other small organics through temperature-controlled elution steps. An additional advantage associated with this process is the high binding site accessibility due to the "surface-imprinting" procedure thanks to the template immobilization.[63, 64] Finally, this solid-phase synthesis is much more rapid than other protocols (e.g. dialysis-based purifications, which can take several days).[46, 60]

In the protocol here adopted, the template-bearing solid phase is suspended in the polymerization mixture composed of NIPAm, BIS, TBAm and AAc, with or without AcrU respectively if Plain or AcrU MIP NPs are being prepared. The polymerization process is then initiated in mild conditions by adding a redox-initiation system (APS and TEMED), optimized for the MIP NPs formation to take place in an aqueous environment, compatible with potential biological templates such as DNA or proteins.[41, 46, 60] The AcrU monomer was incorporated into the MIP matrix at 44 ± 2 % (w/w) of the feed ratio under these conditions. The polymerization was allowed to proceed for 15-20 h at 20 °C. At the end of the polymerization process, the reaction vessel is likely to contain a mixture of high-affinity MIP NPs, low-affinity MIP NPs and unreacted monomer. The template-derivatized glass beads served as affinity media for the subsequent purification stage of the MIP NPs. To purify this mixture, the whole content of the vessel was poured into a SPE cartridge and maintained at 20 °C to allow removal of all the unreacted monomers and other low-affinity materials while keeping high-affinity MIP NPs bound to the immobilized template. The high-affinity products were ultimately eluted from the template-derivatized glass beads by percolating double-distilled H_2O at 60 °C (Figure 1). The increase in temperature disrupts the interactions between the immobilized target and the high-affinity MIP NPs, thus assisting in their elution and collection. The usage of NIPAm as backbone monomer results in the production of thermoresponsive nanomaterials,[60, 65] but from our experience this is not an

issue for the imprinting process, since MIP NPs prepared with this procedure using backbone monomers other than NIPAm (e.g., acrylamide) exhibited poor imprinting performance (data not shown). We speculate that the presence of NIPAm ensures to retain a certain "flexibility" which allows the MIP NPs to properly interact with the templates during the imprinting as well as rebinding processes. Our selection process is based, rather than on shape or size changes of the MIP NPs due to the temperature, on the fact that raising the temperature will increase the rate of exchange of the particles with the template phase, which will assist in eluting the particles, as well as potentially reducing the strength of the association. The overall purification takes about 1 h, and both synthesis and purification could readily be automated.[40, 41]

In accordance with previous results,[40] it was also possible to recycle the template-modified solid phase without drastic variations in yield of high-affinity product among different batches (Table 1; data based on three sequential polymerizations from the same template-modified beads). Given these are high-affinity MIP NPs obtained after a selection process, the obtained yield values are reasonable.[41, 46]

Table 1. Yield of Plain and AcrU MIP NPs imprinted for dA, dG or dC (n = 3).

MIP NPs	Yield (% w/w ± SD)
dA Plain	32.9 ± 1.6
dA AcrU	24.2 ± 2.1
dC Plain	15.3 ± 3.8
dC AcrU	20.2 ± 3.5
dG Plain	24.1 ± 3.8
dG AcrU	30.3 ± 2.2

Transmission Electron Microscopy (TEM) analysis. TEM measurements have shown that MIP NPs exhibited a size of 10-20 nm and a spheroidal shape (see Figure 2 for a typical

sample of NPs made for dA). These results are in agreement with the dynamic light scattering (DLS) data, which exhibited diameter values of 25 ± 5 nm in deionized water for the same type of NPs. This confirms that the presence or absence of the AcrU monomer in the preparation did not affect the size. In addition neither sedimentation nor visible aggregation phenomena were observed during storage (even after two months at 4° C). According to Zeta potential measurements performed on dA Plain and dA AcrU MIP NPs, these latter exhibited a more negative Zeta potential value (-9 mV for dA Plain, -24 mV for dA AcrU, in PBS 0.1 M pH 7.4), which indicates that the nucleoside monomer might also contribute to the stabilization of the MIP NPs dispersion.

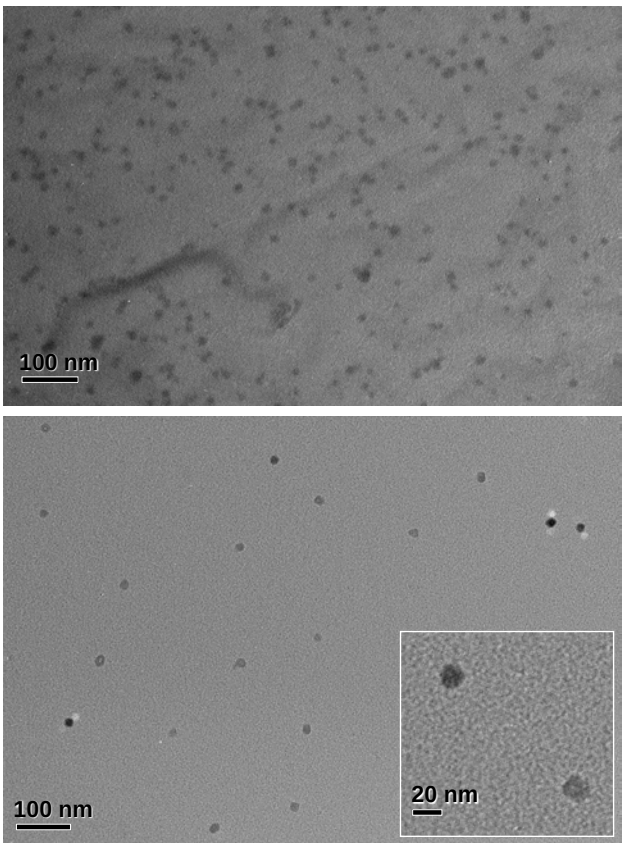


Figure 2. A typical TEM image of Plain MIP NPs (top) and AcrU MIP NPs (bottom) at 30000× magnification. Both MIP NPs were imprinted for dA. Inset details the spheroidal shape of the MIP NPs.

QCM microgravimetric analysis of Plain and AcrU MIP NPs. The affinity and specificity of dA Plain and AcrU MIP NPs was investigated using a Stanford Research Systems QCM200 5 MHz quartz crystal microbalance by immobilizing each template onto the gold crystal surface. The immobilization strategy ensured the presence of a short "spacing" sequence between the surface and the immobilized template, which should prevent steric hindrance as well as mimicking the immobilization conditions on the solid phase used during imprinting. Several concentrations (from of 0.125 to 2 µg/mL) of the high-affinity fraction of dA MIP NPs, either Plain or AcrU, were sequentially flowed (from the lowest to the highest concentration) on the chip bearing one of the templates (either dA, dC or dG), and their binding behavior was recorded. A schematic representation of the QCM assay is represented in Figure 3, while the results of the study are summarized in Figure 4.

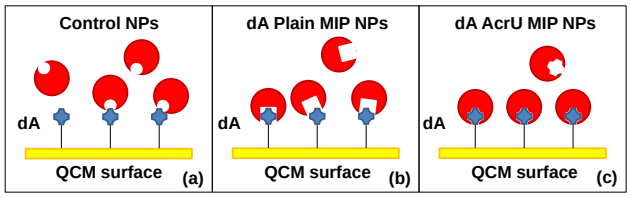


Figure 3. Schematic representation of the binding to immobilized dA of: (a) control NPs (MIP NPs imprinted either for dC or dG); (b) dA Plain MIP NPs; (c) dA AcrU MIP NPs. The introduction of the AcrU monomer improves the recognition performance towards the imprinted template.

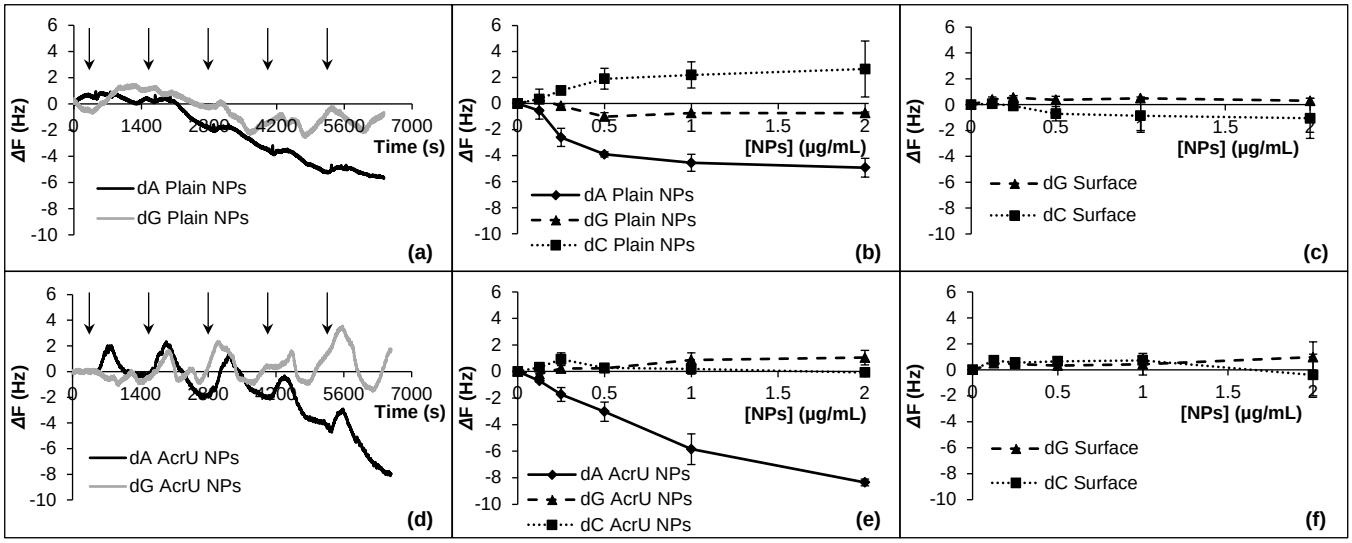


Figure 4. (a) A typical outcome of a Plain MIP NPs QCM experiment on dA-derivatized surface; absorption to dA is characterized by a drop in frequency on exposure to MIP NPs solution. Arrows indicate the points of injection. (b) dA Plain MIP NPs rebinding to complementary dA immobilized template (diamonds, solid line), and dC Plain MIP NPs (squares, dotted line) and dG Plain MIP NPs (triangles, dashed line) non-specific binding to dA immobilized template. (c) dA Plain MIP NPs cross-reactivity test to dC-derivatized (squares, dotted line) and dG-derivatized (triangles, dashed line) surfaces. (d) A typical outcome of an AcrU MIP NPs QCM experiment on dA-derivatized surface; absorption to dA is characterized by a drop in frequency on exposure to MIP NPs solution. Arrows indicate the points of injection. (e) dA AcrU MIP NPs rebinding to complementary dA immobilized template (diamonds, solid line), and dC AcrU MIP NPs (squares, dotted line) and dG AcrU MIP NPs (triangles, dashed line) non-specific binding to dA immobilized template. (f) dA AcrU MIP NPs cross-reactivity test to dC-derivatized (squares, dotted line) and dG-derivatized (triangles, dashed line) surfaces. Concentration of all MIP NPs ranged from 0.125 to 2 μg/mL. QCM measurements were performed in PBS (0.01 M, pH 7.4) at 20 °C. Of note are the "peaks" after injection of the samples (Figure 6a and 6d). These are due to the relative changes in pressure during the injection process. All points are measured after system has

stabilized in buffer after injection. Data for 6b, 6c, 6e and 6f are differences from original starting frequency and were calculated from these stabilized periods. Error bars represent ±1 SD (n = 3)

The sensorgrams presented here show the ability of both dA Plain (Fig. 4a and 4b) and dA AcrU MIP NPs (Fig. 4d and 4e) to recognize and bind their target specifically. The affinity of MIP NPs was assessed in a cross-reactivity study of the dG and dC MIP NPs, both Plain and AcrU towards dA (e.g., dC and dG MIP NPs were injected onto the dA crystals). In all cases, only the NPs imprinted for dA actually bound to dA, with dC and dG NPs not showing any binding neither in the case of Plain nor AcrU MIP NPs (Fig. 4b and 4e).

A peculiar QCM behavior has been observed for dC Plain MIP NPs on dA (Fig. 4b). We hypothesize that the unexpected increase in frequency after the injection of the MIP NPs is caused by poor interactions between the sensitive layer and the MIP NPs, possibly due to the different size of the imprinted sites, which in turn would lead to free movement of particles, thus causing a positive frequency shift (anti-Sauerbrey behavior). This is an unusual but known phenomenon in QCM analysis, and has been extensively studied by the group of Dickert *et al.*[66, 67]

Despite presence of moderate non-specific interactions towards dC from dA MIP NPs (Fig. 4c and 4f), which can be due to the smaller size of this template in comparison with the imprinted dA molecule, there was no evidence of cross-reactivity towards the other purine dG structure, which stresses the good affinity and specificity properties of the produced dA MIP NPs (Fig. 4c and 4f). More importantly, as hypothesized, the introduction of the AcrU monomer into the polymerization mixture increases both the affinity and specificity properties towards the imprinted template (Fig. 4b and 4e).

This increase in affinity and specificity is very interesting in light of the fact that dA, dC and dG are linked to the glass support by their amine groups, likely rendering the base-

pairing face at least partially inaccessible to the normal hydrogen-bonding geometry. This suggests that the specific recognition is likely to be more complex than simple Watson-Crick hydrogen bonding. The base-specific recognition, however, suggests that hydrogen bonding nonetheless plays a role, perhaps occurring in a non-canonical conformation.

Given that these are single bases and base-stacking is not required, non-linear alignment of the bases is a possibility. It would be interesting to test whether an oriented immobilization of the template[61] by immobilizing the sugar portion of the nucleosides and thus maximizing the possibility of establishing Watson-Crick pairing interactions between the template and the polymerizable nucleoside would improve the affinity and specificity of recognition. Likewise, performing an UV polymerization process might result in a higher incorporation of the polymerizable monomer and associated increase in recognition.[38-40] Both these aspects are currently under investigation.

4. Conclusions

We successfully produced MIP NPs for dA by exploiting a modified polymerizable nucleoside (AcrU) as a tailored functional monomer. Nanoparticles were obtained using a solid-phase imprinting polymerization strategy in which template-derivatized glass beads double as an affinity matrix for production as well as selection and purification of synthesized imprinted nanoparticles.

The nanomaterials obtained are spheroidal in shape, with a size comparable to natural antibodies.[68] The solid-phase imprinting strategy guarantees ease of accessibility of the binding sites, resulting *de facto* in a surface-imprinting procedure.

MIP NPs recognized dA specifically when assessed by QCM, while NPs imprinted for dC or dG did not exhibit recognition properties towards dA, even in the presence of the tailored

nucleoside AcrU monomer, thus confirming that the imprinting process is a key step in the formation of the recognition sites.

Currently more studies are being performed in order to assess the effect of the orientation of the templates on the solid phase as well as the effect that different initiation procedures might have on both the incorporation of the polymerizable moiety and the recognition ability of the resulting nanosystems.

We believe that this approach might be further explored to potentially integrate oligomer or longer DNA sequences into MIP matrixes for producing hybrid MIP-DNA materials with improved recognition performance. The gentle polymerization conditions here used should guarantee that the DNA monomers would retain their stability, potentially leading to hybrid DNA-MIP NPs suitable for *in vitro* diagnostic applications or even *in vivo* applications as drug development and delivery systems.[69] We are actively exploring the potential of MIP NPs within these applications.

ASSOCIATED CONTENT

Supporting Information. UV calibration curves for the AcrU monomer, dA, dG and dC templates; sessile water contact angle measurements for the immobilization of nucleoside templates onto QCM crystals. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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